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# Sperm Shape Abnormalities in Carbaryl-Exposed Employees

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Semen was collected from 50 men occupationally exposed to carbaryl (1-naphthyl methyl carbamate) in a production plant for durations of 1 to 18 years and compared to semen from a control group of 34 unexposed, newly-hired workers. Employment, fertility, health, personal data, and blood samples were collected for each individual. Semen samples were analyzed for changes in sperm count, morphology, and frequency of sperm carrying double flourescent bodies (YFF). As a group, the exposed workers showed a significantly higher proportion of sperm with abnormal head shapes than did the control group ($p < 0.005$). Age, smoking habits, and medical problems did not appear to affect this result. This finding appears to be limited to men working in the carbaryl production area at the time of sampling. Sperm count and YFF did not show similar differences, which may be because they are known to be statistically less sensitive to small changes. Formerly exposed workers (away from carbaryl for an average of 6.3 years) showed a marginally significant elevation in sperm abnormalities compared to controls ($p < .05$, one-tailed statistical analyses) suggesting that the increase in abnormal morphology may not be reversible. However, the question of reversibility is sensitive to confounding factors and small sample sizes and, therefore, requires further study.

With these data a definitive link between carbaryl exposure and human seminal defects cannot be established. Although a distinct effect on sperm morphology was seen in the exposed group, the increases in sperm shape abnormalities were not related to exposure dose (estimated by number of years on the job or job classification during the year prior to semen collection). Inexplicably, the increases in sperm abnormalities were seen primarily in currently exposed men who had worked with carbaryl for less than approximately 6 years. These findings suggest the need for further study since other workplace-related factor(s) may be responsible for the elevated sperm abnormalities seen in this study.

## Introduction

1-Naphthyl methyl carbamate, also known as carbaryl or Sevin, is a broad spectrum insecticide. Humans can be exposed to this agent during its manufacture and its widespread application. A clinical study of dermal exposure to carbaryl showed that carbaryl can be readily absorbed through the human skin (*1*). Although dermal absorption appears to vary with anatomical site, the scrotum generally showed high absorption for pesticides.

Though animal studies support the fact that carbaryl reaches the mammalian testes, seminal vesicles, and prostate (*2*), the reported effects of carbaryl on spermatogenesis are inconsistent. Several studies, have shown no testicular effect attributable to carbaryl (*3-6*). However, these studies generally focused on fertility and did not quantitate the effects on germ cells. As reviewed by Whorton et al. (*7*), much of the information on the effects of carbaryl on spermatogenesis comes from the Russian literature (*8-19*). Reported testicular changes include histological changes in the seminiferous epithelium. Semen evaluations showed diminished sperm counts and/or sperm motility. Chronic feeding of carbaryl produces atrophy of the seminiferous tubules, cellular degeneration, necrotic foci, and inflammation of blood vessels in the testes of

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rats (*20*). Chronic oral administration of 3 mg of carbaryl/kg of body weight in rats reduced numbers of spermatogonia and testicular spermatozoa (*21*).

Carbaryl exposure has also been shown to produce abnormal sperm. In rats, Vashakidze et al. (*10*) linked carbaryl ingestion to the production of "malformed" sperm. In mice, the proportion of abnormal and acrosomeless sperm increased almost 10-fold at doses showing no other apparent testicular effects (*22*).

In a study of the effects of carbaryl exposure on human spermatogenesis, Whorton et al. (*7*) measured sperm counts as well as blood levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone in men working in the carbaryl-manufacturing process. When compared to a historical control group of unexposed men from three other studies of workplace exposure, this cohort showed no seminal or blood abnormalities that could be related to carbaryl, although a small increase of possible statistical significance was observed in the proportion of oligospermic men in the exposed group.

The mutagenicity of carbaryl remains ambiguous. It does not seem to be mutagenic in the Salmonella/ microsome assay even with metabolic activation (*23*), nor does it inhibit testicular DNA synthesis in mice (*24*). However, it is reported to be weakly positive in the induction of ouabain resistance in Chinese hamster cells (*25*), to induce unscheduled DNA synthesis in cultured human fibroblasts (*26*), and to be weakly mutagenic in the Drosophila sex-linked recessive lethal test (*27*).

A number of human semen assays have been developed for the study of chemically exposed workers (*28,29*). The approach of using a battery of semen assays increases the sensitivity of detecting chemically induced testicular abnormalities as seen in numerous human studies of exposure including cancer chemotherapeutic drugs (*30*), occupational exposure to dibromochloropropane (*31-33*), occupational exposure to lead (*34*), and antifertility agents (*35*). These studies suggest that changes in sperm count, motility, morphology, and sperm with two fluorescent bodies (YFF test-thought to represent sperm with two Y chromosomes due to meiotic nondisjunction) are all sensitive indicators of exposure of the human testes to chemical agents.

Here we report our findings of seminal analyses in a cohort of carbaryl production workers. We used the same cohort of exposed workers studied by Whorton et al. (*7*). However, we used newly hired workers in the plant as the control group instead of the historical controls used by Whorton. Plant controls were not available at the time of Whorton's study.

# Method of Procedure

## Description of the Cohort

As shown in Table 1, the cohort of exposed men used in this study is virtually the same as that of Whorton et al. (*7*), and where possible used the same semen sample collected by Whorton. All of these men were either current employees at the carbaryl production area or were past workers with at least one year's experience in carbaryl production at the time when Whorton et al. collected the semen samples in July 1978. Our cohort consisted of 101 males and included 52 baggers, 24 operators, and 25 other employees (supervisors, maintenance, etc.) identified from employment records. Of the 101 men, 26 declined to provide a specimen, and 25 had vasectomies. The remaining 50 men provided semen samples for analyses. For a control group, we studied 34 men who gave semen samples as part of their pre-employment medical examinations before assignment to the chemical plant.

## Questionnaire and Medical Examination

A physician interviewed all of the participating men using a standard questionnaire emphasizing employee's work, reproductive, and medical histories and gave each a medical examination focused on the urogenital tract. The purposes of the study and the need to collect blood and semen samples were explained to each man.

## Estimation of Exposure Dose

Available air sampling data were insufficient to provide precise estimates of personnel exposure to carbaryl in the work environments. However, as reviewed by Whorton et al. (*7*), data collected for the company's ongoing industrial hygiene program were available and provided valuable insight on the general range of airborne carbaryl concentrations in the workplace. Both area sampling and personal sampling revealed a wide range of airborne carbaryl concentrations. For example, in the operations area, three samples ranged from 0.36 to 14.21 $mg/m^3$ with a mean of 4.9 $mg/m^3$. In the distribution area, however, the calculated mean of 0.347 $mg/m^3$ seemed much more representative of true conditions, because 22 samples were taken, ranging from 0.03 to 1.8 $mg/m^3$. In this same area, 36 personal samples were taken with a mean of 0.439 $mg/m^3$ and

**Table 1. Participants in semen study.**

| Job[a] | Numbers of men identified from company records | Declined participation | Vasectomies | Numbers of men providing semen samples | Number of men for each semen assay | | |
|---|---|---|---|---|---|---|---|
| | | | | | Counts | Morphology | YFF |
| Exposed men | | | | | | | |
| Bagger | 52[b] | 16 | 14 | 22 | 22 | 22 | 6 |
| Operator | 24 | 2 | 5 | 17[d] | 16[e] | 16[f] | 11 |
| Other | 25[g] | 8[h] | 6 | 11[i] | 10[e] | 11 | – |
| Total exposed | 101 | 26[h] | 25 | 50 | 48 | 49 | 17 |
| New hires | 34 | – | – | 34 | 34 | 34 | 17 |

[a]Job classification in the year immediately prior to semen collection (July 1978) or during the last year of working in the carbaryl production area.

[b]Not 53 as in the study of Whorton et al. (*7*) because one bagger was a foreman in the year prior to semen analyses and was therefore included in the "other" category.

[c]Not 23 as in Whorton's study because of 1 man reclassified as foreman.

[d]Not 16 as in Whorton's study because one sample that was excluded from sperm counts for technical reasons was adequate for our studies on sperm morphology.

[e]One man each excluded for technical reasons as in Whorton's study.

[f]One azoospermic man excluded, but includes another man excluded in Whorton's study for technical reasons.

[g]Not 24 as in Whorton's study (*7*) because this number includes the one bagger we reclassed as a foreman.

[h]Not 9 and 27 as in Whorton's study, because one man who had originally declined participation provided a sample used in our study.

[i]Not 8 as in Whorton's study, because one man was reclassified as foreman (see b), one sample was excluded from Whorton's study for technical reasons but was adequate for our studies on sperm morphology, and one man who had previously declined participation provided a sample used in our study (see h).

a range of 0.0 to 1.8 mg/m$^3$. These doses are well within the ranges that might be expected to cause biological effects. For example, Best and Murray (*36*) saw significant changes in serum cholinesterase activity and urinary excretion of free 1-naphthol (a metabolite of carbaryl) in their carbaryl population exposed to similar concentrations.

We developed an ordinal ranking of exposure groups based on the type of job held during the past year. One year was chosen because semen studies in animals and humans suggest that exposures for a period from approximately 1-2 months up to 1 year before semen sampling are likely to show the maximum effect on seminal abnormalities (*28*). Men were assigned to the following exposure groups: control (new hires), low dose (supervisors, foremen, vacation and sick fill-ins for bagging or operating positions, maintenance personnel and other support staff), and high dose (full-time baggers and operators). We also grouped these men by the number of years they had worked with carbaryl.

## Laboratory Analyses

***Semen.*** Each participant was asked to provide a semen sample by masturbation after observing three days of sexual continence. Morning samples were collected at home in provided glass jars and were delivered to the plant's clinical laboratory. Because the interval between specimen production and delivery could not in every case be held to 2 hr or less, sperm motility, a time-dependent variable, was not recorded. Only one semen sample was collected from each individual. Sperm counts were measured in the plant's clinical laboratory with a hemocytometer and were recorded as number of sperm per milliliter of ejaculate. Ejaculate volumes were also recorded. Smears of sperm were prepared for subsequent microscopic analyses of morphological defects and frequencies of fluorescent bodies. For morphological analyses, air-dried smears were fixed in 95% ethanol and stained by a modified Papanicoloau method (*37*). Five hundred sperm were scored for each individual and classed as shown in Figure 1. All slides were scored blind and compared to a set of standard reference slides at regular scoring intervals. Although interlaboratory comparisons of sperm morphology scores typically show high variability (*38*), within laboratory comparisons can show remarkable reproducibility when the scoring criteria are standardized. For the analyses of the frequency of sperm carrying double fluorescent bodies (YFF), air-dried smears were stained with a quinacrine dihydrochloride method adapted from Pearson et al. (*39*). Smears were fixed for 10 min in Carnoy's, air-dried again, then allowed to soak for 5 min in pH 6.0 (mono- and dibasic sodium phosphate) buffer. The slides were stained for 6 min in 0.2% quinacrine dihydrochloride (in pH 6.0 buffer), rinsed in pH 6.0 buffer, then mounted

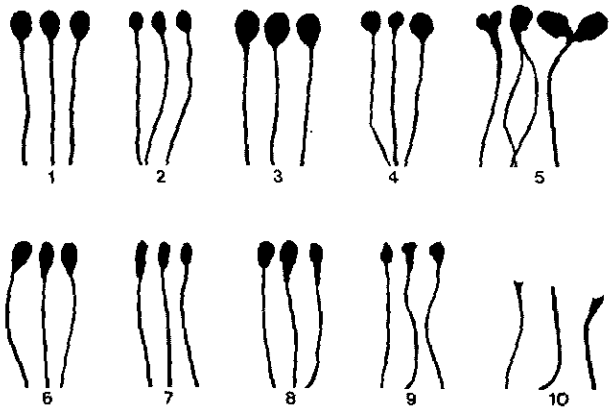


FIGURE 1. Shape variations in human sperm. The head shapes of the sperm in (*1*) are oval, which we consider normal; sperm (*2*) to (*10*) are scored abnormal: (*2*) small, (*3*) large, (*4*) rounded, (*5*) doubles, (*6*) narrow at the base of the head, (*7*) narrow, (*8*) pear-shaped, (*9*) amorphous, and (*10*) ghost-like.

in pH 6.0 buffer. The slides were read under darkfield illumination (*33*) and 500 sperm per sample were scored as OF, (those sperm carrying no fluorescent bodies), 1F, (sperm with one fluorescent body, presumably carrying a single Y chromosome) and 2F, (YFF test, i.e., those sperm with two fluorescent bodies, presumably carrying two Y chromosomes due to errors in meiotic disjunction).

***Blood.*** Blood samples were collected by early morning venipuncture. Only the exposed cohort was studied and only 36 men agreed to venipuncture. The serum was separated, frozen, and sent to the Endocrinology Laboratory, Alta Bates Hospital, Berkeley, California, for analyses of testosterone, FSH, and LH by radioimmunoassay using the methods referenced by Whorton et al. (*7*).

## Statistical Analyses

The data for sperm counts, morphology, and fluorescent bodies (YFF) was analyzed in several ways to determine whether the distribution of data for the carbaryl-exposed men was significantly different from the control cohort of new hires, and whether there was any relationship to intensity or duration of exposure to carbaryl. To determine for each parameter whether the mean value in the carbaryl-exposed men was significantly different from the control cohort we used Cochran's *t*-test for unequal variance (*40*); cumulative frequency plots were compared by using the Kolmogorov-Smirnov two-sample test (*41*). Since we expected seminal changes in one direction only, one-tailed statistical analyses were generally used (*40,42*). The contribution of certain confounding factors such as age (*43*), smoking (*44*), recent illnesses and medical drugs (*35*) were also tested by using multiple linear regression analyses (*40*). Correlation among semen parameters, blood parameters, and other data from the questionnaires were also investigated using correlation analyses (*40*).

# Results

## Comparison of Examination and Interview Data

Exposed workers and controls were compared for differences based on the questionnaire and the physical examination. Table 2 shows that the groups are comparable with respect to smoking, medical illness, and previous exposure to hazardous agents other than carbaryl. Because of the small sample sizes and the small number of children born to the control group, the groups were not compared for differences in reproductive capacity and outcome. The results of the physician's examination did not link any urogenital abnormalities to exposure. The control group was younger than the exposed workers. The mean age (± standard deviation, range)

**Table 2. Characteristics of exposed and control populations.**

| | Exposed (50 men) | Unexposed (34 men) |
|---|---|---|
| Men with confounding factors[a] | | |
| Smokers, more than 1 pack per day for at least 1 year | 11/50 = 22% | 8/34 = 24% |
| Men with positive medical histories | 6/50 = 12% | 5/34 = 15% |
| Smokers with positive medical histories | 3/50 = 6% | 2/34 = 6% |
| Men with previous exposure to other agents | 1/50 = 2% | 3/34 = 9% |
| Total | 17/50 = 34% | 12/34 = 35% |
| Men without confounding factors | | |
| Total | 33/50 = 66% | 22/34 = 65% |

[a]Confounding factors: smoker (smoked more than one pack of cigarettes/day in the last year), positive medical history (unilateral testicular atrophy, presence of epdidymal nodule, recent urinary tract infection, varicocoele, diabetes) or previous exposure to other hazardous agents.

Table 3. Effects of confounding factors and age on sperm counts in control and carbaryl-exposed men.

| | Control | | Exposed | |
|---|---|---|---|---|
| | Number | Sperm count | Number | Sperm count |
| Cohort | 34 | 128.7 ± 23.6[a] | 48 | 140.7 ± 20.3 |
| Men without factors[b] | 22 | 145.8 ± 34.7 | 35 | 130.1 ± 18.6 |
| Men with factors[b] | 12 | 97.4 ± 19.1 | 13 | 146.1 ± 50.6 |
| Men, 18-40 yr old | 33 | 124.7 ± 23.9 | 26 | 120.3 ± 24.0 |
| Men, older than 40 | 1 | 258 | 22 | 151.1 ± 30.5 |
| Men without factors, 18-40 yr old | 22 | 145.8 ± 34.7 | 19 | 143.3 ± 30.6 |
| Proportion of oligospermic males[c] | | 2/34 = 5.9% | | 7/48 = 14.6% |

[a]Average sperm counts in millions per milliliter ± standard error of the mean.
[b]*Confounding factors: smoked more than one pack of cigarettes/day in the last year, had a significant medical problem, or was previously exposed to other hazardous agents.*
[c]Males with less than $20 \times 10^6$ sperm/ml ejaculate.

for the controls was 26.6 yr (± 6.0, 18-44) compared to 40.7 yr (± 10.0, 22-61) for the exposed workers. There was only one (out of 34) control male over 40 years of age, while nearly half the exposed (24 out of 50) were over 40 years of age.

## Assay for Sperm Count

In Figure 2*a* we plot the sperm counts of 34 new hires and 48 carbaryl workers as cumulative distributions. As noted in Table 1, 2 of the 50 semen samples from the exposed group were not included in the calculations of sperm counts because incomplete semen specimens were obtained. Both the Kolmogorov-Smirnov two sample test and the Cochran's *t*-test showed no statistically significant differences between the two distributions or their means. As shown in Table 3, the control and exposed groups were also compared for confounding factors i.e., heavy smoking, previous exposure to other agents, and medical problems, as well as age. Although men with such factors in the control group had a slightly reduced average sperm count when compared to men without these factors, this difference was not statistically significant and is not seen in the exposed group. Comparisons of total sperm per ejaculate also showed no significant differences. Linear regression analyses suggests that age was not significantly correlated to sperm counts in these groups.

No significant effects of carbaryl exposure on sperm counts were seen even when men 18 to 40 yr old without confounding factors were compared with similar controls. However, a slightly higher proportion of oligospermic men (men with less than $20 \times 10^6$ sperm/ml) was found in the exposed group (7/48 = 14.6%) than in the control group (2/34 = 5.9%), but this difference was not statistically significant ($p = 0.1$, one-tailed analyses).

## Assay for Sperm Morphology

In Figure 2*b* the cumulative distributions of sperm shape abnormalities in 34 controls and 49 carbaryl workers are plotted. One of the 50 men from the exposed group was not analyzed because

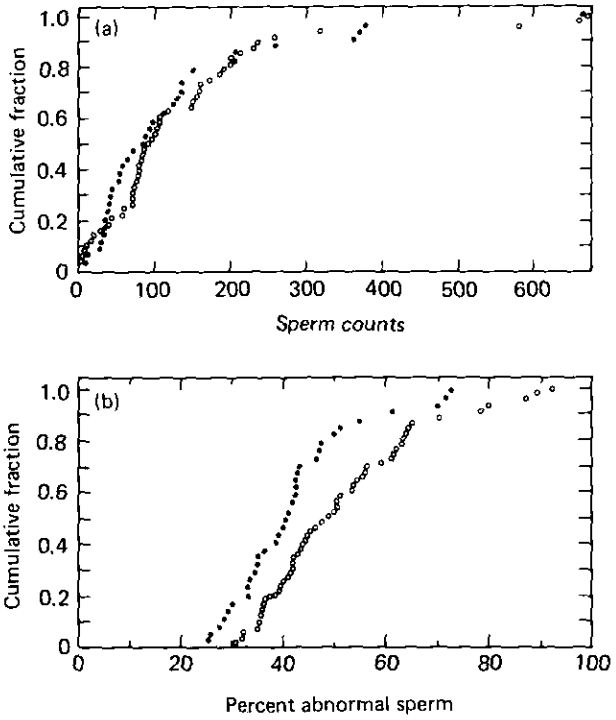


FIGURE 2. Sperm counts (*a*) and sperm morphology (*b*) in control and carbaryl-exposed men. Each point in both panels represents the value for (•) a single control or (o) exposed individual. Sperm counts (*a*) are given in millions of sperm per milliliter in a single ejaculate of each man. Sperm morphology (*b*) represents the frequency of abnormally shaped sperm per individual based on the analyses of 500 sperm per ejaculate. The data for the men in each group are ranked and plotted as cumulative distributions.

of azoospermia (see Table 1) yet the two samples excluded from the analyses of sperm counts were accepted for the analyses of morphology. The distribution of abnormal sperm morphology in the exposed workers is significantly elevated above controls ($p < 0.005$, Cochran's $t$-test). For each semen sample, sperm were assigned to shape categories as shown in Figure 1. The difference in the proportion of abnormally shaped sperm between exposed and control men was not due to an elevation in a specific type of morphological abnormality in sperm.

In Table 4 this difference was analyzed by exposure status (current vs previous), confounding factors, age, and proportion of teratospermic men. None of the confounding factors, including age, significantly affected the proportion of sperm abnormalities in the control or exposed males.

***Previously Exposed Workers.*** Of the 49 exposed men analyzed for sperm morphology, 19 were previously employed in the carbaryl area and presumably were currently working with different chemicals in other areas of the plant. The average time (± standard deviation, range) since employment in the carbaryl area was 6.3 years (± 3.9, 1-12). Previously exposed workers as a group showed a marginally significant ($p< 0.05$, one-tailed) elevation in mean (± SEM) sperm abnormalities (50.0 ± 4.1%) when compared to controls (41.9 ± 2.1%). When men without factors were compared, the sample sizes were reduced and the difference was no longer statistically significant.

***Proportion of Teratospermics.*** As shown in Table 4, when currently and previously exposed men were grouped together and compared to controls, the elevation in the proportion of teratospermic men (14/49 = 28.6% vs. 4/34 = 11.8%) approaches accepted values of statistical significance ($p = 0.06$, one-tailed). We define teratospermic men as those with more than 60% abnormal sperm forms.

***Dose Response.*** To determine whether the elevation in sperm abnormalities was related to exposure dose during the past year, all new hires and currently exposed men were grouped as control, low, or high exposure as described in Methods. The results, plotted in Figure 3, indicate that low and high exposure groups did not differ significantly from each other but were both significantly elevated over the controls.

***Time Response.*** To determine the relationship between sperm abnormalities and years working with carbaryl, we plotted percent abnormal sperm against number of years exposed for the 30 currently exposed workers (Fig. 4). Among the currently exposed there is a significant negative correlation ($r = -0.42$, $p < 0.025$). This compares with the significant negative correlation found between age and percent abnormal sperm ($r = -0.55$, $p < 0.005$) in the same group of currently exposed workers, suggesting that the higher sperm abnormalities are

**Table 4. Effects of exposure status, confounding factors, and age on the percent abnormal sperm in control and carbaryl-exposed men.**

| | Controls | | Currently exposed | | Previously exposed | |
|---|---|---|---|---|---|---|
| | Number | Abnormal sperm, % | Number | Abnormal sperm, % | Number | Abnormal sperm, % |
| Cohort | 34 | 41.9 ± 2.1[a] | 30 | 52.0 ± 2.6<br>$p < 0.005$[b] | 19 | 50.0 ± 4.1<br>$p < 0.05$ |
| Men without factors[c] | 22 | 42.0 ± 2.7 | 21 | 52.8 ± 3.2<br>$p < 0.01$ | 15 | 48.2 ± 4.0<br>N.S. |
| Men with factors | 12 | 41.7 ± 3.7 | 9 | 50.0 ± 4.8<br>N.S. | 4 | 57.1 ± 13.4<br>N.S. |
| Men 18-40 yr old | 33 | 41.8 ± 2.2 | 18 | 57.9 ± 3.4<br>$p < 0.001$ | 7 | 50.9 ± 6.3<br>N.S. |
| Men older than 40 | 1 | 47.0 | 12 | 43.1 ± 2.4<br>N.T. | 12 | 49.5 ± 4.7<br>N.T. |
| Men without factors and 18-40 yr old | 22 | 42.0 ± 2.7 | 14 | 56.2 ± 4.3<br>$p < 0.01$ | 5 | 46.6 ± 5.8<br>N.S. |
| Teratospermic males, %[d] | | 4/34 = 11.8% | | 9/30 = 30% | | 5/19 = 26.3%<br>$p = 0.06$[e] |

[a]Average percent of abnormally shaped sperm ± standard error of the mean.

[b]The probability of significance when compared to the control grouping using Cochran's t-test, one-tailed (*40, 42*), N.S. = not statistically significant, N.T. = not tested.

[c]Confounding factors: smoked more than one pack of cigarettes per day in the last year, had a significant medical problem, or was previously exposed to other hazardous agents.

[d]Males who by our scoring criteria show more than 60% abnormal sperm forms.

[e]Probability of significance when values for currently and previously exposed men are combined using test for comparison of proportions, one-tailed (*40, 42*).

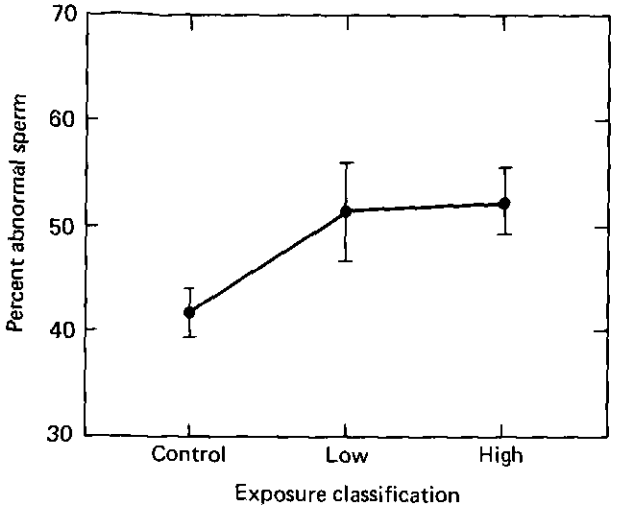


FIGURE 3. Sperm morphology in men currently working with carbaryl grouped by job classification. Each point and error bar represents the mean and standard error of the mean of 34 control, 11 low-dose males and 19 high-dose males. Males were grouped as low or high dose according to the job they held during the year immediately before to semen collection. Only those men working with carbaryl at the time of semen collection were included.

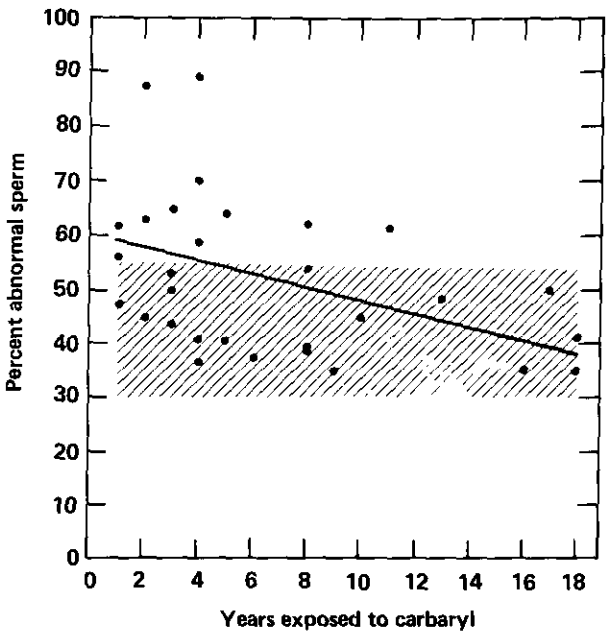


FIGURE 4. Sperm morphology in men vs. number of years working with carbaryl. Each point represents the percent abnormal sperm for one man. Only those men working with carbaryl at the time of semen collection are included. The solid line represents the linear regression line through these data. The shaded region represents the control mean ± one standard deviation.

found in the younger members of the exposed group, mainly those that have worked with carbaryl for less than approximately 6 years.

In previously exposed men, no significant correlations were found among levels of sperm abnormalities, years working with carbaryl, and years since working with carbaryl. These analyses were hampered by small sample sizes.

## Assay for Fluorescent Bodies

A sample of 17 men in the high exposure classification (Fig. 3), i.e., currently exposed baggers and operators, showed 1.0 ± 0.3% of sperm with double fluorescent bodies (2F, Table 5) in comparison to 17 control males who showed 0.8 ± 0.2%. This difference was not statistically significant. The control and exposed men also did not differ in the frequency of sperm with single fluorescent bodies (1F). However, these two groups did differ as expected in sperm morphology ($p < 0.01$).

## Correlations Among Semen and Blood Parameters

No relationships were found among sperm abnormalities, sperm with double fluorescent bodies, FSH, LH, and testosterone. However, a significant correlation ($r = -0.42, p < 0.005$) was found between sperm abnormalities and sperm count in the carbaryl exposed workers. A plot of sperm abnormalities vs. sperm counts of these males suggests that the men with sperm concentrations of less than 80 times $10^6$/ml of semen as a group had more sperm abnormalities than those with over 80 × $10^6$ sperm/ml (18 men, 64.0 ± 3.8% of abnormal sperm vs. 29 men,

**Table 5. Semen characteristics of men scored for sperm with fluorescent bodies, YFF test.**

| | *n* | Sperm counts | Abnormal sperm, % | Sperm with 1F, % | Sperm with 2F, % |
|---|---|---|---|---|---|
| Controls | 17 | 131.9 ± 39.6[a] | 41.2 ± 2.5 | 44.7 ± 0.9 | 0.8 ± 0.2 |
| Exposed | 17 | 130.8 ± 36.5 | 52.6 ± 3.6 | 44.3 ± 1.0 | 1.0 ± 0.3 |
| Significance[b] | | N.S. | $p < 0.01$ | N.S. | N.S. |

[a]Mean ± standard error of the mean.
[b]Cochran's *t*-test one-tailed, N.S. = not significant (*40, 42*).

Table 6. Sample size required to detect a 25% change in mean value over control value.

| | Sperm parameter | | |
|---|---|---|---|
| | Count | Morphology | YFF |
| Assumed distribution | Log-normal | Normal | Poisson[a] |
| Mean | 132 × $10^6$/ml | 41.9% | 0.8% |
| Standard deviation | 160 × $10^6$ | 12.4% | 0.7% |
| 25% increase in standard deviation units | 0.2 | 0.8 | 0.3 |
| Sample size for 5% level test with 90% power[b] | 214 | 26 | 41 |

[a]Normal with square root transform.
[b]See Owen (*45*).

43.6 ± 1.8% abnormal sperm with a $p < 0.001$, Cochran's $t$-test). These correlations suggest that sperm counts as well as sperm morphology may be affected in the carbaryl workers. The negative findings with the assay for sperm counts may be due to the relative statistical sensitivities of these two assays (see Table 6). Comparisons of controls did not show similar correlations.

# Discussion

The results of this study show that workers in a carbaryl-production area had increased levels of morphologically abnormal sperm when compared to controls. Although the elevations in sperm abnormalities were common to currently exposed workers grouped by job classification as low dose and high dose, dose dependence was not found. Neither sperm count nor presence of double-fluorescent bodies were significantly affected in the exposed groups.

We observed a negative correlation between number of years working in the carbaryl area and percent abnormal sperm. We conjecture three possible explanations for this curious relationship. First, the men working longer may be currently exposed less because of seniority; second, some form of biological or pharmacological adaption to exposure may occur (although we don't know of any precedent for this); and third, there may be selection for nonaffected males (for example, older workers who are particularly sensitive to carbaryl and who might have high levels of sperm abnormalities may be switching to other jobs). These conjectures can only be tested by further study of carbaryl workers.

## Reversibility of the Sperm Morphology Effect

The previously and currently exposed workers were compared to controls to determine whether the elevated proportion of sperm abnormalities in the currently exposed might be reversible with time after exposure. The distribution of sperm abnormalities in previously exposed men was marginally elevated above controls ($p < 0.05$). The mean of the distribution of previously exposed men (50.0%) was high and very similar to that of the currently exposed men (52.0%). However, when men with confounding factors were excluded from comparison, the mean of the previously exposed group (46.6%) more closely approached the control mean of 42.0%, the sample sizes were reduced, and the difference is no longer statistically significant. A similar comparison of controls and currently exposed men still showed a statistically significant elevation in the exposed. Both a larger sample size and repeated, periodic semen evaluations in a group of men who have left their jobs in the carbaryl area are needed to properly evaluate the possible reversibility of the sperm morphology effect.

## Effects of Age on Sperm Morphology

The average age (± SD) of the carbaryl exposed group (40.7 ± 10.0 yr) was significantly higher than the control group (26.6 ± 5.6 yr), $p < 0.001$. The possibility arises that the increase in percent abnormal sperm seen in the exposed group is due simply to the increased ages of the men in that group. Schirren et al. (*43*) detected a slight increase with patient age of abnormal forms of sperm, but this was based on men with andrological disorders. In an independent study of 24 healthy men, 18 to 73 years of age, we found no statistically significant correlation between age and percent abnormal sperm, $r = 0.03$ (unpublished data). Also, in the present study there was no statistically significant correlation between age and percent abnormal sperm in the control group ($r = 0.07$). Furthermore, among the exposed carbaryl workers, there was a

statistically significant inverse correlation between age and percent abnormal sperm, ($r = -0.30$, $p < 0.05$), indicating that the younger men had the higher percentage of sperm abnormalities. This is further seen by examining Table 4. The 18 currently exposed men under 40 years of age show 57.9% ± 3.4 abnormal sperm, while those older than 40 years show 43.1% ± 2.4, almost control levels. This difference is highly significant ($p < 0.005$, Cochran's one tailed $t$-test). We conclude that the statistically significant increase in abnormal sperm among the carbaryl exposed group is not due to semen defects resulting from advancing age.

## Statistical Characteristics of the Semen Assays

The different results obtained with assays for sperm counts, morphology and and fluorescent bodies (YFF) may be due to intrinsic differences in their statistical sensitivities. Table 6 shows the sample sizes required to detect a 25% change in the mean value of the controls in our sample (*45*). The sample sizes required depend on the spread of the measurements in the controls (measured by the standard deviations). Thus, if (a) all three sperm assays are equally affected by the perturbing agent(s) and (b) the effect is small, we would have predicted the sperm morphology assay to be most likely to show a statistically significant response.

## Genetic Implication of Findings in Human Semen

Decades of studies on human and animal semen have yielded compelling evidence that sperm can be used to assess testicular function and to diagnose pathology. Males with reduced sperm counts, reduced sperm motility, or increased abnormal sperm shapes are usually less fertile. However, the extent of heritable genetic abnormalities associated with increased sperm anomalies remains unclear. Several lines of indirect evidence on mice and humans support the link between induced sperm abnormalities and heritable genetic effects (*28*, *46*). First, a high correlation exists between agents that induce germ-cell mutations in mice such as dominant lethal mutations and those that induce sperm-shape abnormalities. Second, when male mice exposed to agents that induce sperm abnormalities (such as lead acetate or ionizing radiation) were mated to normal females, an increased proportion of their offspring showed sperm defects. Subsequent studies have shown that sperm changes in these abnormal offspring behave like dominant mutations and that recessive and X-linked genes are also known to be involved in sperm shaping (*28*).

In humans, several studies have shown a link between abnormal embryos and seminal abnormalities in the male parent. In a study by Furuhjelm et al. (*47*) of the Karolinska Institute in Sweden, fathers of 201 spontaneous abortions had reduced sperm counts and marked increases in sperm abnormalities when compared to fathers of normal pregnancies (see Fig. 5). Similar results were obtained in a study by Czeizel et al. (*48*) in 50 husbands of women with two or more spontaneous abortions and 50 men who had fathered normal healthy children. These animal and human studies suggest a genetic link between seminal defects (especially increases in sperm shape abnormalities) and genetic abnormalities in offspring.

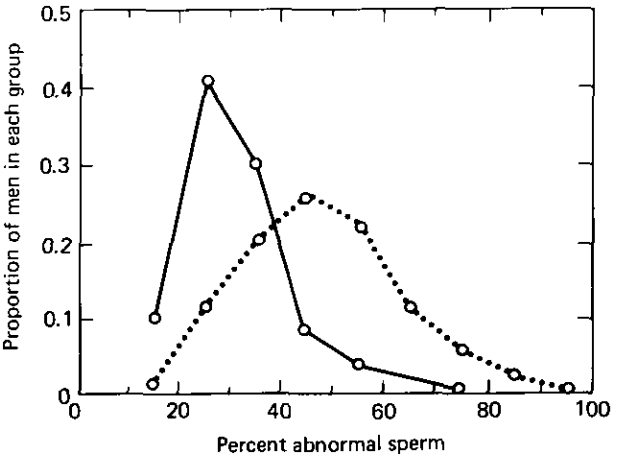


FIGURE 5. Sperm-shape abnormalities in fathers of spontaneous abortions. Dashed line represents the distribution of *sperm-shape abnormalities in 201 fathers of spontaneous* abortions. Data were collected in Stockholm during 1958 to 1961. Solid line represents sperm shape abnormalities in 116 fathers of pregnancies resulting in normal living children. Reproduced from Furuhjelm et al., (*46*).

# Conclusions

Men working in a carbaryl production plant showed higher percentages of sperm shape abnormalities than did new-hires in the plant. This result cannot be explained by differences in smoking habits, illness, medication, or age.

Sperm density and YFF assays did not identify statistically significant differences related to exposure. These assays, however, are shown to be statistically less sensitive than the sperm morphology assay.

For sperm morphology, there was no dose dependence as judged by job classification in the

carbaryl area; however, there was a curious, inverse relationship with the number of years on the job, suggesting that the men working for less than 6 years were most affected.

A comparison of men working with carbaryl at the time of semen collection and those men no longer in the area suggests that the effect on morphology may not be reversible. Since confounding factors and small sample sizes affect this conclusion further human studies are required to fully assess reversibility.

Based on the human data presented, we strongly recommend further animal and human studies with carbaryl to determine to what extent, (a) carbaryl is responsible for the elevated sperm abnormalities seen in the production workers and (b) elevated levels of sperm abnormalities may be related to heritable genetic damage.

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# Use of *Lilium longiflorum*, cv. Ace Pollen Germination and Tube Elongation As a Bioassay For the Hepatocarcinogens, Aflatoxins

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**Although various animal tissues are used for bioassay of aflatoxins ($B_1$, $B_2$, $G_1$, $G_2$), a rapid bioassay dependent upon a plant part's response does not exist. Both pollen germination (G) and tube elongation (TE) were enhanced in a 3.0 *mM* $KH_2PO_4$ (K)-containing but $AFB_1$-lacking, modified Dickinson's medium. The $B_1$ did not affect G when K was withheld but K supplementation impaired G above 15 μg/ml $B_1$. Without K, 5-20 stimulated but 25 and 30 μg/ml $B_1$ inhibited TE which was suppressed by every $B_1$ conc tested in K-containing medium. Addition of $NaH_2PO_4$(N) instead of K to medium did not promote G. Slight G stimulation occurred at 16.6μg/ml mixed aflatoxins (MA) in medium lacking either K or N but low G inhibitions were observed with K or N. The MA at 33.3 μg/ml reduced G 2.5% in K's or N's absence and 26 or 17% in their presence. While K did not stimulate TE without MA, N did 26%. At 16.6 and 33.3 μg/ml MA, TE was reduced 19, 6, 19% and 24, 25, 31%, respectively, in control, K- and N- media. Pollen G and TE were markedly sensitive to $G_1$. Significant inhibitions of *Zea mays* seed G were observed at 5.8 and 11.6 μg/ml $B_1$ but not root elongation (RE) from 0.4-11.6 μg/ml. The MA (31.5 μg/ml) administered for 72-240 hr did not influence either *Arachis hypogeae* seed G or RE. However, imbibing 5 cultivars each of *Avena sativa* (65-117 hr) and *Hordeum vulgare* (39-89 hr) inhibited RE 4/15-62%. Thus, except for *Z. mays*, pollen G and TE appear to be more $B_1$-sensitive than seed G and RE. But, the pollen bioassay is less sensitive than both certain animal bioassays (0.025 μg/ml) and analytical methodologies (10 pg.)**

## Introduction

Although a number of bioassays for aflatoxin $B_1$ ($AFB_1$) and certain other aflatoxins exist which employ a variety of animals (*1*), a bioassay which utilizes a plant or alternatively one or more of its parts has not been developed. However, there are published reports, which have been reviewed by Dashek and Llewellyn (*2*), that describe the effects of aflatoxins on seed germination, seedling growth or elongation, chlorophyll synthesis, enzymatic activities, amino acid uptake, protein or nucleic acid syntheses and cellular ultrastructure of or by a variety of plant parts. A number of these effects could possibly be of service in the development of a bioassay.

Because our initial investigations (*3*) suggested that both *Lilium longiflorum*, cv. Ace pollen germination and tube elongation responded to $AFB_1$ at concentrations as low as 4 μg/ml (approximately 30% inhibition of germination) and 8 μg/ml (about

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25% inhibition of tube elongation), we have attempted to develop a bioassay utilizing these two parameters of pollen growth and development. This paper reports the results of that attempt, an examination of aflatoxin effects on seed germination and elongation of attached roots of certain crop plants, a comparison of the sensitivities of various plant and animal tissues to aflatoxins and a comparison of the sensitivities of the pollen bioassay and analytical methods for quantitating aflatoxins.

# Materials and Methods

## Pollen Germination Conditions

***Experiments with $AFB_1$ and $KH_2PO_4$.*** Two stock solutions of Dickinson's medium (*4*) without tetracycline were adjusted to pH 5.2; the control and experimental stocks lacked and contained 3.0 mM $KH_2PO_4$, respectively. Aflatoxin $B_1$ (A grade, Calbiochem., LaJolla, Calif.) was dissolved in 7 ml of acetone which was then added to 500 ml of experimental medium to yield 30 μg/ml toxin. This concentration was verified by combined thin layer chromatography and a visual dilution technique which is sensitive to 2 ppb (*5*). Acetone (7 ml) was supplied to the control medium prior to autoclaving. The experimental medium was diluted to yield media containing 5, 10, 15, 20 and 25 μg/ml toxin.

*Lilium longiflorum*, cv. Ace pollen (6 months, 4°C) was added in lots of 10 mg fresh weight to 20 ml aliquots of sterile medium in sterile, disposable Petri dishes which were then incubated at 26 ± 2°C for 4 hr. To measure tube lengths and assess percent germination, photomicrographs of the dishes were taken subsequent to positioning the dishes over a partitioned grid. The germination percentages and tube lengths presented in Table 1 are means and standard deviations for three experiments. Each of these experiments had two controls. The total number of grains and tubes which were scored and measured, respectively, was 350 per treatment.

***Experiments with Mixed Aflatoxins and Either $KH_2PO_4$ or $NAH_2PO_4$.*** Because the media which contained or lacked 3.0*mM* $KH_2PO_4$ differed not only in phosphate but also their potassium contents, we examined the possibility that the K cation rather than the $PO_4$ anion either stimulated or inhibited pollen germination and/or tube elongation. Therefore, pollen was germinated in medium with or without 3.0*mM* $KH_2PO_4$ or $NaH_2PO_4$. Mixed aflatoxins (5 μg/ml $AFB_1$, 0.2 μg/ml $AFB_2$, 27.5 μg/ml $AFG_1$ and 0.5 μg/ml $AFG_2$) in chloroform were added to Petri dishes at 16.6 and 33.3 μg/ml. Following evaporation of the chloroform, 20 ml of sterile medium and 20 mg fresh weight lots of pollen were added to the Petri dishes which were incubated at 26 ± 2°C for 4 hr. Then, 1 ml aliquots of a 40% formaldehyde solution were pipetted into the media. Pollen tube lengths within media drops, which were removed at random, were measured with a microscope equipped with an ocular micrometer. The experiment was repeated twice and within each experiment there was a replicate for each treatment. Although the total number of pollen grains scored for percent germination of each

**Table 1. Germination and tube elongation for pollen sown in medium containing or lacking $AFB_1$ and $KH_2PO_4$.[a]**

| $AFB_1$ Concentration, μg/ml | Tube length, μm | | | | Germination, % | | | |
|---|---|---|---|---|---|---|---|---|
| | With $KH_2PO_4$ | % Change | No $KH_2PO_4$ | % Change | With $KH_2PO_4$ | % Change | No $KH_2PO_4$ | % Change |
| 0 | 812 ± 53 | — | 757 ± 22 | — | 44.3 ± 3.8 | — | 29.5 ± 2.8 | — |
| 5 | 795 ± 14 | − 0.9 | 814 ± 29 | + 7.5 | 45.9 ± 2.8 | + 3.6 | 34.0 ± 2.2 | +15.3 |
| 10 | 727 ± 37 | −10.5 | 865 ± 17 | +14.3 | 46.4 ± 15.5 | + 4.7 | 38.7 ± 4.7 | +31.2 |
| 15 | 770 ± 47 | − 5.2 | 882 ± 49 | +16.5 | 39.6 ± 7.7 | −10.6 | 32.7 ± 2.9 | + 7.5 |
| 20 | 793 ± 14 | − 2.3 | 857 ± 17 | +13.2 | 41.5 ± 6.1 | − 6.3 | 35.1 ± 4.7 | +19.0 |
| 25 | 622 ± 33 | −23.6 | 729 ± 33 | − 3.7 | 32.2 ± 2.6 | −27.3 | 30.9 ± 2.4 | + 4.7 |
| 30 | 519 ± 33 | −36.1 | 673 ± 33 | −11.1 | 20.0 ± 1.3 | −45.1 | 29.7 ± 2.5 | + 0.7 |

[a]Two stock solutions of Dickinson's (4) medium but without tetracycline were adjusted to pH 5.2; one stock lacked 3.0*mM* $KH_2PO_4$ (control) and the other (experimental) contained it; $AFB_1$ (grade B, dried *in situ*, Calbiochem, LaJolla, Ca.) was dissolved in 7 ml acetone and then added to 500 ml of the experimental medium with a resulting $AFB_1$ concentration of 30 μg/ml; subsequent to autoclaving, $AFB_1$ concentration was verified by thin layer chromatography coupled with a visual dilution technique which is sensitive to 2 ppb; 7 ml of acetone were also added to the control medium prior to autoclaving; the 30 μg/ml $AFB_1$ experimental medium was diluted to yield media which contained 5, 10, 15, 20 or 25 μg/ml; 10 mg fresh weight lots of stored (6 months, 4°C) *Lilium longiflorum*, cv. Ace pollen were sown in 10 ml aliquots of sterile medium in sterile, disposable, plastic Petri dishes; pollen was incubated for 4 hr at 27 ± 2°C. To obtain tube lengths and percent germinations, photomicrographs were made of the Petri dishes following their positioning over a 3 mm grid; data tabulated from the photomicrographs are means and standard deviations for three experiments; each experiment had two controls; 350 pollen grains were scored and tubes measured per treatment; % change equals the % change from the control; a variant of this table and figure legend will appear (*10*).

treatment varied, that number approximated 3000. The total number of tubes measured was 260.

***Experiments with $AFB_2$, $AFG_1$ and $AFG_2$.*** The specificity of the pollen bioassay was examined by dissolving aflatoxin $B_2$ ($AFB_2$) aflatoxin G, ($AFG_1$) and aflatoxin $G_2$ ($AFG_2$) in acetone with subsequent transfer to Petri dishes of aliquots which yielded 15 and 30 μg/ml upon addition of medium which either contained or lacked 3.0*mM* $KH_2PO_4$.

## Seed Germination Conditions

Fifty *Arachis hypogaea*, cv. Florigiant seeds were treated with Botec fungicide, rinsed with sterile $H_2O$ and then sown in lots of five per Petri dish on three layers of sterile standard laboratory paper toweling. The seeds were arranged such that their embryo ends pointed toward the center of the dish. To each dish 15 ml of sterile $H_2O$ containing 31.5 μg/ml mixed aflatoxins (5.6 μg/ml $AFB_1$, 0.2 μg/ml $AFB_2$, 25.0 μg/ml $AFG_1$ and 0.8 μg/ml $AFG_2$) were added. The dishes were sealed with parafilm and tilted to an angle which permitted the roots to elongate in a straight fashion. The seeds were incubated in the dark at 24 ± 2°C for 72, 144, 168 and 240 hr when percent germination and root lengths were measured, the latter with a mm rule.

The same experimental design was also used for both *Avena sativa* and *Hordeum vulgare* seeds except that five cultivars of each genera were tested. These cultivars were: Coker, Norline, Moregrain, Windsor and Roanoke for *A. sativa* and Surry, Henry, Volbar, McNair and Barsoy for *H. vulgare*. Other differences in the design included 10 seeds and 10 ml of test solution per dish. The germination times were 65, 89 and 177 hr for *A. sativa* and 29, 63 and 89 hr for *H. vulgare*.

*Zea mays* seeds were surfaced sterilized with 10% Chlorox for 10 min, at which time they were rinsed with 300 ml distilled $H_2O$. Seeds in lots of 15 were sown on two layers of sterile filter paper and imbibed as above except that the imbibition medium included 50 μg/ml chloramphenicol and the dishes were not tilted. The experiment was replicated three times with duplicate dishes for each treatment within an experiment.

## Statistical Analyses

The data were analyzed by a two-tailed *t*-test for evaluating the difference between population means.

## Preparation of Mixed Aflatoxins for Seed Germination Studies

Mixed aflatoxins were prepared according to Llewellyn et al. (*6*) through inoculation of coconuts with *Aspergillus parasiticus*, strain NRRL 2999. The inoculated coconuts were extracted with cloroform and the extract analyzed for aflatoxins by the combined thin layer chromatography and visual dilution technique. The choloroform extract contained 1270 μg mixed aflatoxins (225 μg/ml $AFB_1$, 6 μg/ml $AFB_2$, 1000 μg/ml $AFG_1$ and 39 μg/ml $AFG_2$). A 50 ml portion of this crude extract was transferred to 1 liter of warm sterile distilled water which was gently heated to drive off the chloroform. This solution contained 31.5 μg/ml mixed aflatoxins in the ratio of individual aflatoxins reported above and was stored in the dark at 8°C.

# Results and Discussion

## Does $AFB_1$ Inhibit Pollen Germination and Subsequent Tube Elongation When $KH_2PO_4$ Is Withheld or Included in the Growth Medium?

Germination was not inhibited when pollen was sown in medium lacking phosphate but containing 5-30 μg/ml $AFB_1$ (Table 1). In contrast, pollen germination was inhibited 10.6, 6.3, 27.3 and 45.1% upon addition of 15, 20, 25 and 30 μg/ml $AFB_1$ to an incubation medium containing 3.0 *mM* $KH_2PO_4$. In the absence of $KH_2PO_4$, only 25 and 30 μg/ml $AFB_1$ inhibited tube elongation. However, this elongation was inhibited at every $AFB_1$ concentration when $KH_2PO_4$ was added to the germination medium. Maximum inhibition occurred at 25 (24%) and 30 (36%) μg/ml.

## Do Mixed Aflatoxins Inhibit Pollen Germination and Tube Elongation When Different Phosphate Salts are Withheld or Added to the Growth Medium?

Both percent germination and tube lengths for pollen sown in medium containing or lacking mixed aflatoxins and either $KH_2PO_4$ or $NaH_2PO_4$ are shown in Table 2. Without phosphate, 16.6 μg/ml and 33.3 μg/ml mixed aflatoxins stimulated and reduced percent germination by 7.8 and 34.7%, respectively. When 3.0 *mM* $NaH_2PO_4$ was included in the medium, the percent germination inhibitions were 17% (16.6 μg/ml) and 16.5% (33.3 μg/ml). In contrast, addition of 3.0 *mM* $KH_2PO_4$ to the incubation medium yielded germination inhibition percentages of 8.3% and 25.6% at 16.6 and 33.3 μg/ml, respectively. Tube elongations for pollen germinated in the absence of phosphate but presence of 16.6 or 33.3 μg/ml mixed aflatoxins were reduced 18.9% and 23.7%, respectively. When the medium was provided

Table 2. Germination and tube elongation for pollen sown in medium containing or lacking aflatoxins and either $KH_2PO_4$ or $NaH_2PO_4$.[a]

| Aflatoxin concentration, μg/ml | Tube length, μm | | | Germination, % | | |
|---|---|---|---|---|---|---|
| | With $KH_2PO_4$ | With $NaH_2PO_4$ | No $PO_4$ | With $KH_2PO_4$ | With $NaH_2PO_4$ | No $PO_4$ |
| 0.0 | 826 ± 198 | 1081 ± 219 | 860 ± 265 | 65.3 ± 20.6 | 53.8 ± 20.4 | 53.2 ± 17.4 |
| 16.64 | 773 ± 97 | 879 ± 147 | 697 ± 131 | 59.9 ± 22.6 | 44.6 ± 14.4 | 57.4 ± 17.7 |
| 33.28 | 616 ± 110 | 748 ± 156 | 656 ± 154 | 48.6 ± 21.2 | 44.9 ± 16.9 | 38.9 ± 26.0 |

[a]Mixed aflatoxins at 16.64 and 33.28 μg/ml (5 μg/ml $AFB_1$, 0.2 μg/ml $AFB_2$, 27.5 μg/ml $AFG_1$, 0.58 μg/ml $AFG_2$) dissolved in chloroform were added to sterile Petri dishes; following evaporation of the chloroform, 20 ml Dickinson's medium (*4*) minus tetracycline and 20 mg fresh weight of stored (1 month, 4°C) pollen were added to each dish; pollen was germinated 4 hr at 26 ± 2°C in the dark; 1 ml aliquots of 40% formaldehyde were added to the media at 4 hr; tube lengths within drops selected at random were measured with a microscope equipped with an ocular micrometer; the experiment was replicated three times with a replicate for each of the above treatments within an experiment; the number of tubes measured per treatment was 260 and the number of grains scored for determining % germination was variable per treatment but approximated 3,000; data are means and standard deviations; a variant of this table and its legend will appear (*10*).

with 3.0*mM* $NaH_2PO_4$, tube elongation was suppressed by 18.7% at 16.6 and 30.8% at 33.3 μg/ml mixed aflatoxins. The percent inhibitions of tube elongation were 6.4% (16.6 μg/ml) and 15.4% (33.3 μg/ml) for pollen germinated in medium containing 3.0*mM* $KH_2PO_4$.

## How Specific Is the Response of Pollen to $AFB_1$?

A comparison of the effects of $AFB_2$, $AFG_1$ and $AFG_2$ on both germination and tube elongation is shown in Table 3. Whereas sowing pollen in medium lacking 3.0*mM* $KH_2PO_4$ but containing 15 μg/ml $AFB_2$ did not significantly inhibit percent germination, 30 μg/ml suppressed germination by 29% and tube elongation by 15% (Table 3). Both of these reductions were statistically significant at 95% confidence level. In contrast, both 15 and 30 μg/ml $AFG_1$ in medium lacking $KH_2PO_4$ suppressed germination by 97.5 and 99.7%, respectively, and tube elongation by 100%. When $KH_2PO_4$ was added to the medium, 15 μg/ml $AFG_1$ decreased germination and tube elongation by 88 and 55%, respectively. However, 30 μg/ml $AFG_1$ impaired germination by 99.7% and tube elongation by 100%.

The addition of 15 or 30 μg/ml $AFG_2$ to media which either contained or lacked 3.0*mM* $KH_2PO_4$ did not inhibit germination except at 30 μg/ml together with $KH_2PO_4$, but, tube elongation was suppressed 34 and 26% at 15 and 30 μg/ml, respectively, for medium which was not supplemented with $KH_2PO_4$. Both suppressions were significant at the 95% confidence level. When the medium was provided with 3.0*mM* $KH_2PO_4$, a 12% (significant) reduction in tube elongation was observed. Therefore, both lily pollen germination and tube elongation appear to be more sensitive to $AFG_1$ than $AFB_1$, $AFB_2$ or $AFG_2$. However, germination experiments with $AFG_1$ which would employ pollen of high viability should be performed to substantiate the results reported here for pollen of low viability. In addition, it is desirable to assess the ability of lily pollen to germinate and elongate tubes at < 15 μg/ml $AFG_1$.

## Do Mixed Aflatoxins Affect Seed Germination and/or Root Elongation of Peanuts, Corn, Barley and Oats?

Inclusion of mixed aflatoxins at 31.5 μg/ml in the imbibition medium was without a significant effect on either germination of *Arachis hypogaea* seeds or elongation of their roots at 72, 144, 168 and 240 hr of imbibition (Table 4).

The lack of an aflatoxin effect on either seed germination or elongation of attached *Arachis hypogaea* roots is somewhat surprising, since an aflatoxin incidence rate of 19% in consumer peanut products has been reported for the United States and Canada during the years 1972–1975 (*7*). This unexpected result is coupled with another. Aflatoxin $B_1$ inhibits the germination and elongation of both attached and excised *Glycine max*, cv. Essex roots (*8-10*), but field-grown soybeans are relatively resistant to invasion by *Aspergillus flavus* (*11*). Aflatoxin was detected in only 2 of 866 soybean samples analyzed by the Department of Agriculture and at total aflatoxin amounts of 10-11 μg/kg.

When *Zea mays* seeds were imbibed in mixed aflatoxins over the concentration range of 0.36-11.60 μg/ml, the percent inhibitions of seed germination were 9.5, 6.0, 13.1, 22.6 and 25% of 0.36, 1.45, 2.90, 5.80 and 11.60 μg/ml mixed aflatoxins, respectively (Table 5). Only the inhibitions which occurred at 5.8

**Table 3. Comparison of the effects of $AFB_2$, $AFG_1$ and $AFG_2$ on *Lilium Longiflorum*, cv., Ace pollen germination and tube elongation.[a]**

| Aflatoxin type | Aflatoxin concentration, μg/ml | With $KH_2PO_4$ | | Without $KH_2PO_4$ | |
|---|---|---|---|---|---|
| | | Germination, % | Tube length, mm | Germination, % | Tube length, mm |
| $AFB_2$ | 0 | | | 30.7 ± 12.0 | 7.5 ± 2.9 |
| | 15 | | | 31.2 ± 6.8 | 7.1 ± 0.3 |
| | 30 | | | 21.8 ± 8.8 | 6.4 ± 2.8 |
| $AFG_1$ | 0 | 20.6 ± 10.3 | 5.9 ± 2.3 | 8.0 ± 5.1 | 4.6 ± 2.5 |
| | 15 | 2.5 ± 0.9 | 2.8 ± 1.6 | 0.2 ± 0.3 | 0.0 ± 0.0 |
| | 30 | 0.08 ± 0.7 | 0.0 ± 0.0 | 0.03 ± 0.06 | 0.0 ± 0.0 |
| $AFG_2$ | 0 | 13.5 | 6.6 | 12.1 ± 4.9 | 6.9 ± 3.2 |
| | 15 | 15.1 | 7.4 | 19.2 ± 8.4 | 4.6 ± 2.7 |
| | 30 | 14.6 | 5.8 | 6.4 ± 7.5 | 5.1 ± 2.5 |

[a]Lots of stored (6 months, 4°C) *Lilium longiflorum*, cv. Ace pollen (20 mg fresh weight) were added to 10 ml Dickinson's medium with or without 3.0*mM* $KH_2PO_4$ and 15 or 30 μg/ml $AFB_2$, $AFG_1$ or $AFG_2$; pollen was germinated in sterile Petri dishes at 26°C for 4 hr when 1 ml of formaldehyde was added to the dishes; drops were removed at random and % germination and tube lengths measured with an ocular micrometer; data are means and standard deviations of four replicates for the $AFB_2$ and $AFG_1$ treatments and six replicates for $AFG_2$ without phosphate; data for $AFG_2$ with phosphate are averages for two experiments; the number of grains scored was 2300-2900 and tubes measured were 300 per treatment; statistical analyses indicated that the differences in mean germination percentages and tube lengths between toxin-treated and untreated pollen were significant in every case.

**Table 4. Effect of aflatoxin on *Arachis hypogaea* seed germination and root elongation.[a]**

| Time, hr | Germination, % | | Root length, mm | |
|---|---|---|---|---|
| | No aflatoxin | With aflatoxin | No aflatoxin | With aflatoxin |
| 72 | 4 | 6 | 4.0 ± 0.0 | 3.3 ± 1.2 |
| 144 | 26 | 30 | 13.7 ± 11.0 | 13.0 ± 7.2 |
| 168 | 28 | 36 | 21.6 ± 16.7 | 17.7 ± 10.1 |
| 240 | 40 | 48 | 34.5 ± 15.9 | 34.1 ± 21.2 |

[a]One hundred peanut seeds were treated with Botec fungicide; 50 seeds were treated with aflatoxin and 50 seeds with $H_2O$; seeds were arrayed in a straight line on laboratory paper toweling in sterile Petri dishes so that the embryo ends pointed toward the dish's center; 15 ml of sterile $H_2O$ containing 31.5 μg/ml mixed aflatoxins or 15 ml $H_2O$ were added to each dish; the dishes were sealed with parafilm and tilted to obtain an angle which allowed the roots to elongate linearly in order to facilitate measurement; seeds were germinated for the above times in the dark at 26°C when root lengths were measured with a mm rule; root data are means and standard deviations for seeds in ten dishes (five seeds/dish).

and 11.6 μg/ml were statistically significant. As for the effects of mixed aflatoxins on *Zea mays* root elongation, 0.36, 1.45, 2.90 and 5.80 μg/ml stimulated this elongation by 6.1, 30.6, 38.8 and 12.2%. Only the stimulation at 2.90 was statistically significant. There was no effect of 11.60 μg/ml mixed aflatoxins on root elongation. The observed suppressions of seed germination at both 5.8 and 11.6 μg/ml are disturbing, since there are a number of reports which demonstrate that ears of corn can be contaminated with the toxin (*12-16*). The incidence of aflatoxins in corn and corn meal for Southeastern United States in 1969, 1970 and 1974 was 41% (*7*). In this connection, during a single week in August of 1977, 78% of the preharvest corn samples from a 31 county survey in Georgia contained over 100 ppb total aflatoxins (*16*).

Imbibition of seeds of various *Avena sativa* cultivars in a solution containing 31.5 μg/ml mixed aflatoxins yielded the following root inhibitions: 32.3, 43.3 and 66.7% (cv., Norline), 4.3, 33.1 and 68.6% (cv., Windsor), 20.5, 37.2 and 65.8% (cv., Coker), 23.4, 46.2 and 68.8% (cv., Moregrain) and 30.3, 42.4 and 61.8% (cv., Roanoke) at 65, 89 and

**Table 5. Effect of aflatoxin on *Zea mays* germination and root elongation.[a]**

| Aflatoxin concentration, μg/ml | Germination, % | Root length, mm |
|---|---|---|
| 0 (control) | 84 ± 10.5 | 4.9 ± 0.40 |
| 0.36 | 76 ± 9.9 | 5.2 ± 0.40 |
| 1.45 | 79 ± 7.1 | 6.4 ± 0.53 |
| 2.90 | 73 ± 8.5 | 6.8 ± 0.60 |
| 5.80 | 65 ± 15.4 | 5.5 ± 0.50 |
| 11.60 | 63 ± 8.2 | 4.8 ± 0.42 |

[a]Seeds were surfaced sterilized in 10% Chlorox for 10 min and then rinsed five times in 300 ml distilled $H_2O$ prior to sowing; seeds in lots of 15 were placed in sterile Petri dishes containing a single layer of filter paper; 10 ml of aflatoxin solution was added to each dish, and the dishes were wrapped and allowed to incubate 40 hr at 24°C; at 40 hr, percent germination and root elongation were determined by using a dissecting microscope; to measure uptake 1 ml germination medium was removed from each dish prior to and following germination; the data are means and standard deviations of three experiments with duplicate dishes for each treatment within an experiment.

117 hr of imbibition, respectively (Table 6).

The effects of 31.5 μg/ml mixed aflatoxins on seed germination and root elongation of various *Hordeum vulgare* cultivars are summarized in Table 7. The percent inhibitions of root elongation were 22.4, 38.0 and 59.7% (cv., Surry), 36.5, 42.6 and 61.4% (cv. Barsoy), 35.2, 47.8 and 58.0% (cv., Volbar), 26.8, 36.7 and 61.2% (cv., McNair) and 15, 36 and 62.2% (cv. Henry) for seeds imbibed 39, 63 and 89 hr, respectively.

As for the incidence of aflatoxins in small grains in "commercial channels" in the United States

**Table 6. Effect of aflatoxin on seed germination and root elongation of various cultivars of *Avena sativa*.[a]**

| *Avena sativa* cultivar | Time, hr | Germination, % | | Root length, mm | |
|---|---|---|---|---|---|
| | | No aflatoxin | With aflatoxin | No aflatoxin | With aflatoxin |
| Norline | 65 | 64 | 70 | 10.5 ± 2.6 | 7.1 ± 1.8 |
| | 89 | 64 | 76 | 23.0 ± 4.8 | 13.0 ± 5.3 |
| | 117 | 67 | 76 | 68.0 ± 11.5 | 22.6 ± 6.0 |
| Coker | 65 | 88 | 80 | 6.8 ± 1.8 | 5.4 ± 1.5 |
| | 89 | 94 | 82 | 17.2 ± 4.7 | 10.8 ± 4.7 |
| | 117 | 94 | 82 | 74.0 ± 8.2 | 25.3 ± 4.0 |
| Windsor | 65 | 72 | 70 | 4.6 ± 1.7 | 4.4 ± 1.2 |
| | 89 | 84 | 76 | 12.1 ± 3.4 | 8.1 ± 2.9 |
| | 117 | 84 | 76 | 71.0 ± 10.9 | 22.1 ± 3.8 |
| Moregrain | 65 | 94 | 82 | 8.1 ± 2.8 | 6.2 ± 2.3 |
| | 89 | 96 | 94 | 21.4 ± 5.8 | 11.5 ± 4.1 |
| | 117 | 96 | 94 | 74.9 ± 8.6 | 23.3 ± 3.6 |
| Roanoke | 65 | 92 | 74 | 8.8 ± 2.8 | 5.9 ± 1.8 |
| | 89 | 94 | 84 | 19.3 ± 6.0 | 11.1 ± 3.6 |
| | 117 | 94 | 86 | 65.3 ± 9.2 | 24.8 ± 5.0 |

[a]Seeds of *Avena sativa*, cvs. Norline, Windsor, Coker, Moregrain and Roanoke were treated, sown in Petri dishes, incubated and percent germination quantified and root lengths measured as in Table 4 except that the incubation times were 65, 89 and 117 hr; the differences in root lengths between treated and nontreated seeds of every cultivar were statistically significant at all times tested except for cultivar Windsor at 65 hr.

**Table 7. Effect of aflatoxin on seed germination and root elongation of various cultivars of *Hordeum vulgare*.[a]**

| *Hordeum vulgare* cultivar | Time, hr | Germination, % | | Root length, mm | |
|---|---|---|---|---|---|
| | | No aflatoxin | With aflatoxin | No aflatoxin | With aflatoxin |
| Surry | 39 | 62 | 40 | 5.8 ± 2.1 | 4.5 ± 1.6 |
| | 63 | 78 | 56 | 21.1 ± 5.8 | 13.1 ± 3.8 |
| | 89 | 78 | 60 | 52.4 ± 7.0 | 21.1 ± 3.7 |
| Barsoy | 39 | 92 | 90 | 6.3 ± 4.0 | 4.0 ± 1.4 |
| | 63 | 92 | 90 | 18.9 ± 4.8 | 10.8 ± 3.0 |
| | 89 | 94 | 90 | 42.2 ± 7.8 | 16.3 ± 3.9 |
| Volbar | 39 | 96 | 94 | 7.1 ± 2.5 | 4.6 ± 1.4 |
| | 63 | 96 | 100 | 24.5 ± 3.8 | 12.8 ± 2.5 |
| | 89 | 96 | 100 | 44.4 ± 6.3 | 18.9 ± 4.1 |
| McNair | 39 | 40 | 34 | 5.6 ± 2.0 | 4.1 ± 1.4 |
| | 63 | 54 | 42 | 22.3 ± 7.1 | 14.1 ± 4.3 |
| | 89 | 56 | 46 | 45.1 ± 10.7 | 17.5 ± 5.8 |
| Henry | 39 | 80 | 78 | 6.0 ± 2.1 | 5.1 ± 2.1 |
| | 63 | 86 | 86 | 25.0 ± 7.2 | 16.0 ± 3.2 |
| | 89 | 90 | 86 | 51.6 ± 6.5 | 19.5 ± 4.7 |

[a]Seeds of *Hordeum vulgare*, cv. Surry, Barsoy, Volbar, McNair and Henry were treated, sown in Petri dishes, incubated and percent germination quantified and root lengths measured as in Table 4 except that 10 seeds and 10 ml of test solution were added per dish and the imbibition times were 39, 63 and 89 hr; the differences in root lengths between treated and non-treated seeds of every cultivar were statistically significant at all times tested with the exception of cultivar Henry at 39 hr.

during the years 1968-1975, 3 of 416 oat and 0 of 254 barley samples examined contained $AFB_1$ (*7*).

## Are Pollen and Seed Germinations Equally Sensitive to Aflatoxins?

Because both the types of the exogenously supplied aflatoxins and probably their uptakes are variable for the plant systems summarized in Table 8, it may be somewhat misleading to compare the effects of aflatoxins on percent germination of various seeds with those of pollen. However, when such a comparison is made, it is apparent that concentrations above 30-50 μg/ml are usually required to impair imbibition in a solution containing 5.8 and 11.6 μg/ml $AFB_1$ for 18 hr resulted in 40 and 80% germination except for *Glycine max*. In the latter, inhibitions of seed germination. In contrast, when the medium was supplemented with 3.0*mM* $KH_2PO_4$, administration of 25 and 30 μg/ml $AFB_1$ for 4 hr reduced pollen germination by 27.3 and 45.1%. However, mixed aflatoxins consisting primarily of $AFG_1$ do not inhibit germination either with or without supplementing the medium with $KH_2PO_4$. This suggests that one reason for the failure of investigators to observe an effect of aflatoxin on seed germination at rather high aflatoxin concentrations could have resulted from the use of mixed aflatoxins rather than pure $AFB_1$.

**Table 8. Summary of the effects of aflatoxins on seed germination.**

| Plant | Effect | Investigator(s) |
|---|---|---|
| *Lepidium sativum* | No impairment at 1, 2, 5, and 10 μg/ml; 35, 90, and 100% inhibition at 25, 50 and 100 μg/ml, respectively | Schoental and White (*17*) |
| Variety of seeds | Variable % inhibition (as high as 100 in *Phalaris canariensis*) at 20 μg/ml | Jacquet et al. (*18*) |
| *Lactuca sativa* 30 cultivars | No inhibition as high as 1,000 μg/ml in one cultivaor nor by 100 μg/ml in the 29 other cultivars | Crisan (*19*) |
| Species of *Cruciferae* (19 plants belonging to 11 species) | No effect at 100 μg/ml | Crisan (*20*) |
| *Vigna sinensis* | 100% inhibition above 50 μg/ml | Adekunle and Bassir (*21*) |
| *Glycine max*, cv. Essex | Inhibitions were 5, 20, 40 and 80 or 6, 4, 13 and 19% for seeds exposed 18 and 36 hr respectively to 0.38, 2.90, 5.80 and 11.60 μg/ml $AFB_1$ | Jones et al (*10*) |
| *Onoclea sensibilis* | Inhibition of spore germinations was 6.7, 7.8, 27.0, 32.6, and 43.8% at 0.78, 1.56, 2.34, 3.13 and 3.90 μg/ml $AFB_1$; this same concentration series yielded mean protonemal cells of 2.55, 2.4, 1.5, 1.3 and 1.4 and 3.0 for the control | Cahill et al. (*22*) |
| *Arachis hypogaea* | No significant effect of 31.5 μg/ml mixed aflatoxins at 72, 144, 168 and 240 hr | Dashek et al. (present work) |
| *Avena sativa* | No significant effect of 31.5 μg/ml mixed aflatoxins at 65, 89 and 117 hr for cvs. Norline, Windsor or Moregrain; 20% inhibition for cvs. Coker and Roanoke at 89 and 117 hr | Dashek et al. (present work) |
| *Hordeum vulgare* | No significant effect of 31.5 μg/ml mixed aflatoxins at 39, 63 and 89 hr for cvs. Barsoy, Volbar and Henry; 17-23% inhibitions for Surry and McNair at 39, 63 and 89 hr, respectively | Dashek et al. (present work) |
| *Zea mays* | No effect at 0.36, 0.73, 1.5 and 2.9 μg/ml; 23% reduction at 5.8 and 11.6 μg/ml | Dashek et al. (present work) |
| *Lilium longiflorum*, cv. Ace pollen | No inhibitory effect from 5-30 μg/ml $AFB_1$ without 3.0 mM $KH_2PO_4$; 10.6, 27.3 and 45.1% inhibition at 15, 25 and 30 μg/ml plus 3.00*mM* $KH_2PO_4$ | Jones et al (*10*); Dashek et at (present work) |
| | No inhibitory effect at 16.64 or 33.28 (5 μg/ml $B_1$, 0.2 μg/ml $B_2$, 27.5 μg/ml $G_1$, 0.58 μg/ml $G_2$) mixed aflatoxins | |

## Is Pollen Tube Elongation More or Less Sensitive to Aflatoxins Than the Elongations of Tissues on a Variety of Plants?

Table 9 demonstrates that aflatoxin concentrations > 30 μg/ml are required to inhibit the elongation of tissues of the majority of those plants thus far examined. This comparison may be more meaningful than that for germination since $AFB_1$ was the aflatoxin of choice in most of the investigations summarized in Table 9. However, data on toxin uptake for the various systems are not available. Given this limitation, it appears that the elongation of *Lilium longiflorum*, cv. Ace pollen tubes is no more sensitive to aflatoxins than that of a variety of plant tissues. Furthermore, tube elongation is considerably less responsive to $AFB_1$ than *Onoclea sensibilis* protonemal development and *Glycine max* root elongation.

## Is the Pollen Bioassay as Sensitive to Aflatoxins as the Commonly Employed Animal Bioassays?

Comparison of the summaries for the effects of aflatoxins on germination (Table 8) and elongation (Table 9) of lily pollen with the effects on various animal systems (Table 10) reveals that pollen bioassay is less sensitive than the commonly employed animal bioassays.

**Table 9. Summary of the effects of aflatoxins on growth and elongation of various plants.**

| Plant | Effect | Investigator(s) |
|---|---|---|
| *Caralluma frerei* | 30 ppm similar to nontreated, death and prevention of growth of upper leaves and floral buds at 100 and 300 ppm | Reiss (*23*) |
| *Phalaris canariensis* | Radicle elongation impaired by as much as 100% at 50 μg/ml mixture of aflatoxins | Jacquet et al. (*18*) |
| *Lepidium sativum* | No inhibition of hypocotyl elongation at 1 μg/ml $AFB_1$, 14.2 and 58.0% inhibition at 10 and 100 μg/ml; no inhibition of radicle elongation at 1 μg/ml $AFB_1$; 23.9% inhibitions at 10 and 100 μg/ml | Reiss (*24*) |
| *Chlorella pyrenoidosa* | Inhibition of growth of four strains by $AFB_1$ at 1 μg/ml | Sullivan and Ikawa (*25*) |
| *Glycine max*, cv. Essex | Nontreated excised roots followed a sigmoidal growth curve with a dry wt. increase from 100% (0 hr) to 108.5% (24 hr); 4.5% dry wt. decline at 4 hr increase to 101.5% (8 hr); and decrease to 99% (12 hr) at 20 μg/ml $AFB_1$ | Young et al. (*26*) |
| *Kalanchoe diagremontiana* | Inhibition of root elongation by approximately 50% at 100 μg/ml $AFB_1$ | Reiss (*27*) |
| *Glycine max*, cv. Essex | % inhibition of attached root elongation was 14 (48 hr) and 26 (140 hr) at 2.9 μg/ml $AFB_1$; 21 (48 hr) and 35 (140 hr) at 5.8 μg/ml; and 36 (48 hr) and 50 (140 hr) at 11.6 μg/ml | Jones et al (*10*) |
| *Arachis hypogaea* | No significant effect of mixed aflatoxin at 31.5 μg/ml on root elongation at 72, 144, 168 and 240 hr | Dashek et al. (present work) |
| *Avena sativa* five cultivars | Percent inhibition of root elongation at 31.5 μg/ml mixed aflatoxins ranged from 4.3 to 68.8% for 5 cvs. imbibed 65 and 117 hr. respectively | Dashek et al. (present work) |
| *Hordeum vulgare* | Percent inhibition of root elongation at 31.5 μg/ml mixed aflatoxins ranged from 22.4 to 62.2 for 5 seeds imbibed 39 and 89 hr. respectively | Dashek et al. (present work) |
| *Zea mays* | Mixed aflatoxins at 0.36, 1.45, 2.90 and 5.80 μg/ml stimulated root elongation by 6.1, 30.6, 38.8 and 12.2% | Dashek et al. (present work) |
| *Lilium longiflorum* | Maximum inhibition of elongation occurred at 25 (23%) and 30 (36%) μg/ml $AFB_1$ when 3.0*mM* $KH_2PO_4$ was added to the germination medium | Jones et al. (*10*) |
| | Elongation inhibited 18.9% and 23.7% by 16.64 and 33.28 μg/ml mixed aflatoxins | |

Table 10. Animal bioassays for aflatoxins $B_1$.

| Concentration | System | Response | Investigator(s) |
|---|---|---|---|
| 0.5 μg/ml $AFB_1$, 24 hr | *Artemia salina* | 90% mortality | Abedi and McKinley (*28*) |
| 1.0 μg/ml $AFB_1$, 24 hr | | 61% " | |
| 0.048 μg/ml $AFB_1$, air cell injection 21 days | Chicken embryo | $LD_{50}$ | Verrett et al. (*29*) |
| 0.025 μg/ml $AFB_1$, yolk injection | | " | |
| 18.2 μg $AFB_1$/50g ducklings | Ducklings | Oral 7 day $LD_{50}$ | Kraybill as cited in Brown (*1*) |
| 16.6 μg $AFM_1$/50g ducklings | | " | |
| 39.2 μg $AFG_1$/50g ducklings | | " | |
| 62.0 μg $AFM_2$/50g ducklings | | " | |
| 84.4 μg $AFB_2$/50g ducklings | | " | |
| 17.2 μg $AFB_2$/50g ducklings | | " | |
| 0.05 to 40 μg $AFB_1$/ml for 3-5 hr | *Bankia setaceae* | Treated-single cell containing many nuclei, control-two or more cells | Townsley and Lee (*30*) |
| 1 μg/ml for 30 min | *Brachydanio rerio* | Abnormal movement of larvae within 30 min; moribund in 5 to 6 hr; yolk sphere darkening within 20 hr; larval death 24 to 36 hr | Abedi and McKinley (*28*) |
| 1 ppb $AFB_1$ in diet | Shasta strain rainbow trout | Liver cancer | Sinnhuber et al. (*31*) |
| 8-20 ppb $AFB_1$ in diet | | *Visible hepatomas, 4-6 months* | |
| Immersion of embryo for 60 min in $AFB_1$ at 0.5 μg/ml | | High incidence of liver cancer some months later | |
| 4 ppb $AFB_1$ | *Salmo gairdneri* | 25 and 48% hepatocellular carcinoma incidence at 9 and 12 months | Masri et al. (*32*) |
| 20 ppb $AFB_1$ | | 56 and 83% incidences at 8 and 12 months | |

## Is the Pollen Bioassay as Sensitive as the Available Analytical Methods for Quantitating Aflatoxins?

Comparison of the data in Tables 8 and 9 with those in Table 11 shows that the pollen aflatoxin bioassay is not as sensitive as the current analytical methodology for quantifying aflatoxins.

## Does the Pollen Bioassay System Have Any Utilitarian Value?

The value of this system is in its rapidity and inexpensiveness as well as the fact that one does not need sophisticated instrumentation and a highly trained technician to perform the bioassay. Furthermore, pollen germination and subsequent tube elongation are especially sensitive to $AFG_1$. However, these advantages are offset by the lack of sensitivity of the bioassay. We suggest that *Onoclea sensibilis* spores and/or *Glycine max* seeds be adopted as organisms of choice for the development of those aflatoxin bioassays which would employ plants.

## Is the Pollen Bioassay as Sensitive to Aflatoxins as the Commonly Employed Animal Bioassays?

Comparison of the summaries for the effects of aflatoxins on germination (Table 8) and elongation (Table 9) of lily pollen with the effects on various animal systems (Table 10) reveals that the pollen bioassay is less sensitive than the commonly employed animal bioassays.

**Table 11. Comparison of sensitivities of analytical methodology for quantifying aflatoxins.**

| Technique | Sensitivity | Error | Reference |
|---|---|---|---|
| Thin layer chromatography | | | |
| Plus visual identification of $B_1$, $B_2$, $G_1$ and $G_2$ by fluorescence intensity | 0.3-0.4 ng | 20-28% | Coomes et al (*33*) |
| plus fluorodensitometry | 0.25-1.5 × $10^3$ μg | ? | Ayres and Sinnhuber (*34*) |
| Plus silica gel minicolumn cleanup and identification of $B_1$ and $M_1$ with TFA | 0.05 μg/kg | Recoveries $B_1$ 85% $M_1$ 65% | Van Egmond (*35*) |
| Plus silica gel cleanup with addition of citric acid to extracting solvent and ammonium sulfate to the extract solution; methanol substituted for acetone during elution from silica gel | 0.1-0.2 ng, $B_1$ and $M_1$ added | Recoveries 81 ± 6 $B_1$ (0.1 μg added) 91 ± 17 $B_1$ (0.2 μg added) 66 ± 12 $M_1$ (0.1 μg added) 82 ± 19 $M_1$ (0.2 μg added) | Truckess and Stoloff (*36*) |
| Plus laser fluorometric determination | 10-1000 pg | rMS% error 26 | Diebold et al. (*37*) |
| High pressure liquid chromatography | | | |
| Waters Associates Model 440 absorbance detector | 10 ng for $B_1$ and $B_2$ | Recovery of $B_1$ and $B_2$ 90-95% | Pons and Franz (*38*) |
| Shimadzu Model RF-510LC spectroflurometer | Relationship between fluorescence peak area and the amount injected linear in the range of 0.3 ng to 120 ng | ? | Manabe et al. (*39*) |
| Fluorescence detection with a packed silica gel flow cell | 0.1 ng | ? | Blanc (*40*) |
| Oscillopolarographic traces | 0.3- 50 μg for $B_1$ and $G_1$ | ? | Garjan et al. (*41*) |
| Ultraviolet light absorption spectrophotometry for $B_1$ | 3-10 μg | | Nabney and Nesbitt (*42*) |
| Sephadex gel filtration G-10 elution with 1% aqueous method | 10 μg | 10% | Manabe et al. (*43*) |

## Is the Pollen Bioassay as Sensitive as the Available Analytical Methods for Quantitating Aflatoxins?

Comparison of the data in Tables 8 and 9 with those in Table 11 shows that the pollen aflatoxin bioassay is not as sensitive as the current analytical methodology for quantifying aflatoxins.

## Does the Pollen Bioassay System have any Utilitarian Value?

Experimental data indicate that pollen systems are not a particularly useful bioassay for aflatoxin hepatocarcinogenicity.

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